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ORIGINAL RESEARCH

Identification and Characterization of a Vancomycin Intermediate-Resistant *Staphylococcus haemolyticus* Isolated from Guangzhou, China

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Background: *Staphylococcus haemolyticus* is an opportunistic pathogen that belongs to coagulase-negative *Staphylococci* (CoNS). Increasing infection and multi-drug resistance cases caused by this strain have been reported and thus it poses a great health threat.

Methods: The third-generation sequencing technology was performed on a *S. haemolyticus* SH-1 isolated from a clinical sample to analyze the drug resistance genes, which included vancomycin resistance related genes. In addition, antimicrobial susceptibility tests, transmission electron microscopy and Triton X-100 stimulated autolysis were conducted to understand its biological characteristics.

Results: The study shows that this clinical isolate is a vancomycin intermediate-resistant strain. Genome comparison also revealed that WalK(N70K) and WalK(R280Q) mutations may contribute to the vancomycin resistant phenotype. Besides, *S. haemolyticus* SH-1 exhibit common features of thicker cell wall and decreased autolytic activity.

Conclusion: *S. haemolyticus* SH-1 with WalKR mutations shows typical characteristics of vancomycin resistant strains. Combining the genome features and biological properties, our findings may provide important information for the understanding of the molecular mechanism of *S. haemolyticus* to vancomycin intermediate-resistance.

Keywords: *Staphylococcus haemolyticus*, drug resistance gene, vancomycin intermediate-resistance, whole genome sequencing

Introduction

Coagulase-negative *Staphylococci* (CoNS), a kind of bacteria colonizing on body surfaces, oral cavities and intestinal tracts, is considered to be no pathogenicity originally. Comparing with *Staphylococcus aureus*, the virulence of CoNS is much lower and it produces free coagulase. However, since 1970s, with increased CoNS infections reported, CoNS has been recognized as one of the important opportunistic pathogens that cause many nosocomial infections. At present, CoNS often causes infections, particularly the patients with low immunity and using indwelling and implantable medical devices.[1–4] In human infections, *Staphylococcus epidermidis, Staphylococcus haemolyticus* and *Staphylococcus saprophyticus* are the most common CoNS.[5] The antibiotic resistance of CoNS poses a big concern on human health because most of CoNS show a high adaptability to the host and hospital environment and exhibit high multidrug resistance (MDR).[6,7] Because of the widespread use of penicillin and methicillin in the last century, CoNS have been found resistant to β-lactam antibiotics and methicillin in most of hospitals.[3,8] In some medical institution, methicillin-resist CoNS may reach 70–92% and the cross-drug resistance is also serious.[9–11] *S. haemolyticus* is a kind of CoNS and its carrier rate ranks only second to that of *S. epidermidis*. Vancomycin is a kind of cationic glycopeptide antibiotic and is an important drug for treating MRSA and MRSE infections.[12] However, with the increased applications of vancomycin in recent

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years, bacteria completely resistant or intermediately resistant to vancomycin have been found and the reported cases are increasing. In our preliminary work, we recovered a *S. haemolyticus* from a clinical sample of a patient. We found that the strain was intermediately resistant to vancomycin. Therefore, drug-resistant CoNS is certainly a critical challenge for clinical therapy.

In the present study, we performed an analysis of antibiotic susceptibility and whole genome sequencing to gather more information about the strain of intermediately vancomycin-resistant *S. haemolyticus*. This study could allow us to understand more about the biological and genetic characteristics of vancomycin intermediate-resistant *S. haemolyticus*. The information obtained may be helpful to explore the mechanism of vancomycin intermediate-resistance in the future.

Materials and Methods

Bacterial Strains and Growth Conditions

The *S. haemolyticus* strain SH-1 was isolated from a blood sample of a 20-year-old patient with extremely severe aplastic anemia by our group in 2018. Strain SH-2 was isolated from the pus sample. Strain XN108 is a clinical VISA isolate and contains a WalK(S221P) mutation. XN108 has typical features of VISA strains and is used in this study as a control for reducing the vancomycin susceptibility.

The blood samples were collected in FA/FN bottles (BioMérieux Co., Ltd.). Blood culture bottles were incubated in an automated BacT/Alert 3D system. After the blood culture became positive, 10 μL of fluid was plated on Columbia blood agar (CBA), and *S. haemolyticus* isolate SH-1 was cultured. The pus sample was inoculated on CBA at 37 °C for 24 h, and *S. haemolyticus* isolate SH-2 was cultured. The isolates SH-1 and SH-2 were identified by VITEK ® MS (BioMérieux Co., Ltd.). The overnight cultures of *S. haemolyticus* SH-1 and SH-2 and *S. aureus* XN108 were diluted into an optical density at 600 nm of 0.05 in a 6-well plate contained 3 mL of fresh TSB. Then, the mixtures were incubated in TSB medium at 37 °C for 12 h. Bacterial cell growths were monitored by measuring the OD_{600} every hour and the growth curves were plotted. The experiments were repeated for at least three times.

DNA Extraction and Whole Genome Sequencing

The isolate was cultured on blood agar and incubated overnight at 37 °C. Then, a single bacterial colony was transferred into the tryptone soybean broth and incubated at 37 °C for 12–16 h. Genomic DNA was extracted using Bacterial Genome DNA rapid extraction kit (Tiangen biotech Co LtD, Beijing).

Genomic DNA was sequenced using PacBio Sequel sequencing system. The third generate data were assembled using Microbial Assembly (smrtlink8), HGAP4 software (smrtlink8) and Canu (v1.6). The coding sequences (CDS) of bacterial DNA were predicted using Glimmer (v3.02), tRNA genes using tRNAscan-SE (v2.0) and rRNA genes using RNAmmer. Pathogenic Island and potential horizontal gene transfer were detected via Island Path-DIOMB software. Prophage structure in genome was predicted by PhiSpy software (v2.3). All predicted protein sequences were Blast with the basic annotated databases including NR, COG/KOG, GO, SwissPort and KEGG.

Antimicrobial Susceptibility Testing

The antibiotic susceptibility test was analyzed using VITEK 2 COMPACT automatic microbiological analysis system and Test Kit GP67 (BioMérieux, France). Minimum inhibitory concentration (MIC) of vancomycin and teicoplanin were determined using E-test analysis method. Procedures and judgements of results were referred to the guidelines of the Clinical and Laboratory Standards Institute (CLSI M100 2021).[13]

Molecular Typing, Phylogenetic and Single-Nucleotide Polymorphism (SNP) Analysis

Seven house-keeping genes (*arc, SH_1200, hemH, leuB, SH_1431, cfxE, Ribose_ABC*) were chosen and submitted to PubMLST database (https://pubmlst.org/organisms/staphylococcus-haemolyticus), revealing the sequence type of SH-1.

Genomes of representative *S. haemolyticus* isolates were downloaded from NCBI RefSeq database. We constructed maximum likelihood phylogenetic tree using Parsnp (https://github.com/marbl/parsnp) according to the Blast of core SNP, drawing phylogenetic tree via iTOL by input genome of SH-1 and the reference genome. Illumina readings of the

test strain were mapped to the reference genome sequence of *S. haemolyticus* strains using Burrows-Wheeler Aligner (BWA) software. SAWtools was used to view and sort the reads of maps. Before running FreeBayes to identify genetic variations, the Picard tool was used to remove repeated reads from the respective alignment sequences. VCFtools was used to filter the data to obtain initial SNP result. The mutated genes were further submitted to NCBI database for Blast with all the data of *S. haemolyticus* in the database, confirming SH-1 mutation sites.

Transmission Electron Microscopy (TEM)

The preparation and examination of samples with TEM were performed according to the following steps as previously described.[14] The bacterial cells in exponential phase were fixed in 2% glutaraldehyde and then were treated with 1% osmium tetroxide for 2 h. Next, the samples were dehydrated with different concentrations of ethanol and embedded. The thin sections were stained and then observed with a transmission electron microscope. Cell wall thickness of strains were determined by transmission electron microscope (JEM1400Plus). Morphometric assessment of cell wall thickness was performed by using image photographs with a final magnification of 80,000×. We measured the thicknesses of cell walls for 20 cells of each strain with nearly equatorially cut surfaces. The data were expressed as the mean ± SDs. Differences were evaluated by Student's *t*-test, where $P<0.05$ was considered statistically significant.

Triton X-100 Stimulated Autolysis

Autolysis experiments were performed as previously described with modifications.[15] Briefly, the overnight cultures of isolates were diluted into tryptone soybean broth and incubated at 37 °C with shaking at 200 rpm. The mid-exponential growth bacterial cells were centrifuged and washed with cold PBS. The bacteria cells were then collected by centrifugation and then were resuspended in 0.1%(v/v) Triton X-100 in PBS buffer. The autolysis was measured every 30 min at OD_{600} at 37 °C with shaking at 200 rpm. The percentage of the optical density at each time point was plotted. Autolysis experiments were performed at least three times.

Results

Whole Genome Characteristics and Gene Compositions

The chromosome of *S. haemolyticus* SH-1 is a 2,622,719 bp circle with 32.85% GC content (Figure S1). The total length of *S. haemolyticus* SH-1 gene is 2,244,669 bp, accounting for 85.59% of the genome length. Predictions for non-coding RNA show that *S. haemolyticus* SH-1 genome contains 63 tRNAs, six 5s rRNAs, five 16s rRNAs, and five 23s rRNAs. In addition, tandem repeat sequence software predicted 125 repetitive sequences with a length of 64,308 bp. 14 CRISPR sequences were predicted using MinCED (v0.3.2). 10 gene islands with a total length of 175,053 bp were predicted in SH-1 by IslandPath-DIOMB software. PhiSpy (v2.3) software was used to predict the prophage structure in the genome. A total of 2 prophage structures were predicted. The whole genome sequence and annotation were deposited in NCBI GenBank under SRA accession number SRR18682786.

We used the CARD database to annotate drug resistance genes. It showed that 190 genes in SH-1 were associated with drug resistance. SH-1 carries the methicillin resistance gene *mecA*, penicillin resistance gene *blaZ*, erythromycin and clindamycin resistance genes *msrA, msrC, ermD*, aminoglycoside resistance genes *aph(3')-IIIa, aad(6), adeR, aac(6')-Ie-aph(2')-Ia*, quinolone resistance genes *patB, norB*, and mupirocin resistance gene *mupA*.

Growth Characteristics

Strains with different vancomycin susceptibility showed distinct growth states. VISH SH-1 and VISA XN108 have slower growth rate compared with vancomycin susceptible *S. haemolyticus* strain SH-2 in the exponential growth period. Both VISH SH-1 and VISA XN108 were found reaching a higher value at the plateau phase (Figure 1).

Antimicrobial Susceptibilities

S. haemolyticus SH-1 exhibited resistance to penicillin, oxacillin, erythromycin, clindamycin, gentamicin, moxifloxacin, trimethoprim-sulfamethoxazole (SXT), levofloxacin, ciprofloxacin. It also exhibited intermediate-resistance to vancomycin

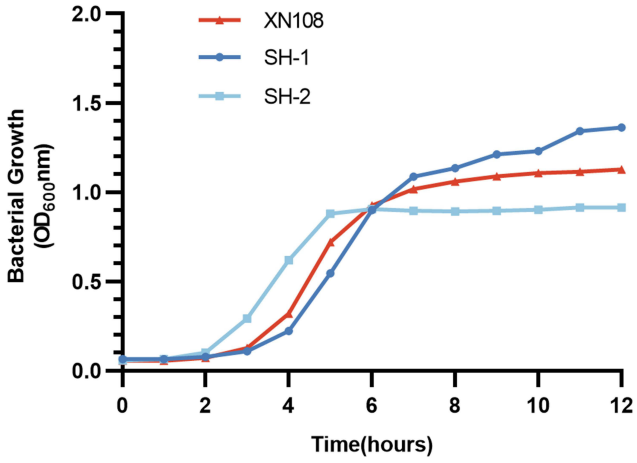

Figure 1 Growth curves of SH-1, SH-2 and control XN108.

and teicoplanin (Table S1). *S. haemolyticus* SH-1 was susceptible to dalfopristin, linezolid, rifampicin and tetracycline. In addition, Cefoxitin screening tests and induced clindamycin resistance tests of SH-1 were found positive. The vancomycin and teicoplanin MIC values were also determined by E-test method. The MIC for SH-1 against vancomycin was 8 μg/mL. And, SH-1 exhibited an increased teicoplanin MIC of 16 μg/mL (Figure S2). According to CLSI criteria,[13] the results demonstrated that SH-1 was intermediate-resistant to both vancomycin and teicoplanin.

Molecular Typing Analysis

Molecular typing analysis reveals that sequence typing of SH-1 is ST97. A total of 161 *S. haemolyticus* isolates were sequenced in PubMLST database and 51 of which were from China. Both SH-1 and SYSUSHA_9 of human respiratory samples isolated from Guangdong province in the database belong to ST97 type.

Phylogenetic and SNP Analysis

Evolutionary analysis shows that SH-1 and VB19458 (GCF_003596365.3) are related in evolution (Figure 2). VB19458 is a linezolid-resistant and vancomycin-susceptible strain isolated from blood sample and it belongs to ST3. SNP analysis of SH-1 strain was then performed using VB19458 as the reference strain. It reveals that there are 150 non-synonymous mutation sites in SH-1. These mutation sites were compared with all *S. haemolyticus* data in NCBI database by further blast alignment analysis. A total of 18 genes (*walk, cobA, mspA, rplB, rpsC, rplX, thrS, ruvB, lepA, grpE, recN, qoxD, uvrA, norA, rplA, nusG, rsmA, metE*) were mutated, and the point of mutations, WalK (N70K) and WalK (R280Q), may be closely related with antimicrobial resistance of vancomycin.

WalK Mutation Sites in SH-1

Whole genome sequencing and SNP analysis results show that WalK (N70K) and WalK (R280Q) mutations on SH-1 may be associated with the vancomycin intermediate-resistance phenotype. We designed primer pairs for PCR assay to determine whether these two mutations were present on SH-1. The PCR amplification products were sent for sequencing. The results showed that, compared with the evolution related isolate VB19458, both WalK (N70K) and WalK (R280Q) mutations were present on the SH-1 genome (Figure S3).

SH-1 Showed Thicker Cell Wall and Decreased Autolytic Activity

Previous studies reported that cell wall thickening was a common feature of VISA strains, as well as the decreased autolytic activity and virulence. We thus performed transmission electron microscopy and Triton X-100 induced autolysis assays to verify whether VISH SH-1 with WalK mutations would show these characters. SH-1 and the control strains were observed under a transmission electron microscope. As expected, both SH-1 and XN108 showed significantly

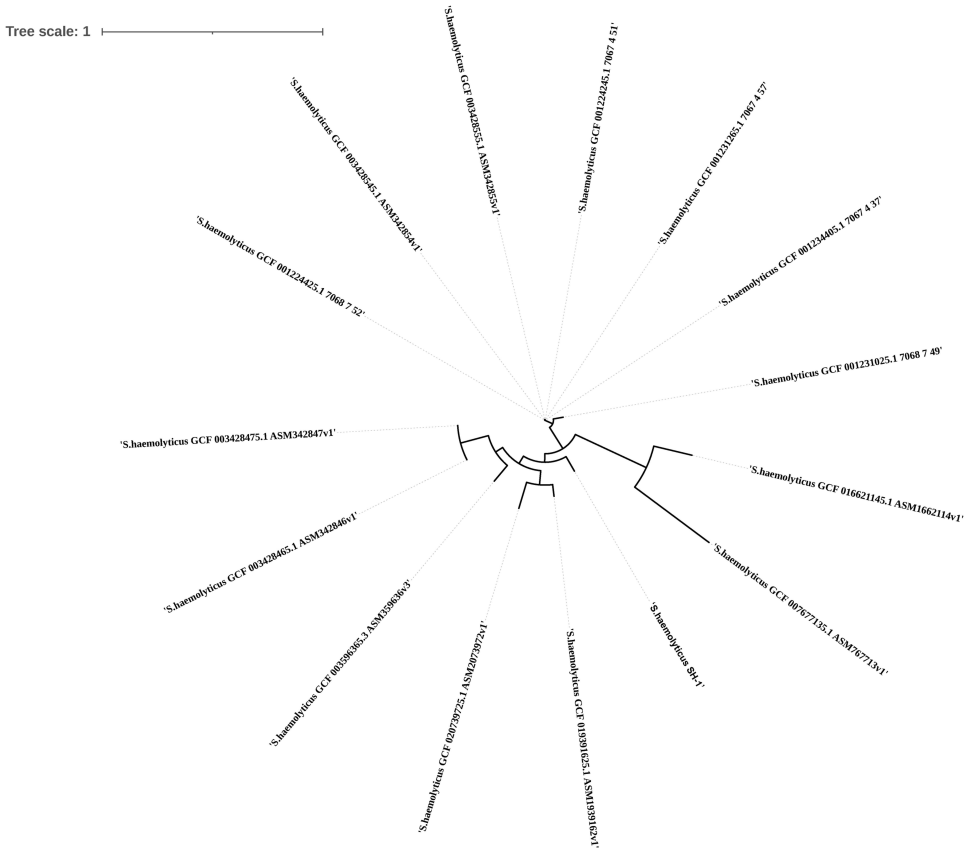

Figure 2 Genome-wide evolutionary map of *Staphylococcus haemolyticus* SH-1.

thicker cell walls (45.28±4.27 nm and 44.80±4.06 nm, respectively) than that of the vancomycin susceptible strain SH-2 (26.46±3.21nm). The Triton X-100-induced autolysis assay also showed that both SH-1 and XN108 exhibited reduced autolytic activity compared to the strain SH-2 (Figure 3).

Discussion

Coagulase negative *Staphylococci* has become common and significant pathogenic bacteria in clinical.[16] The strain may cause respiratory tract infection, blood infection, urinary tract infection and other clinical diseases.[17–19] *S. haemolyticus* has a very broad spectrum of drug resistance among clinical CoNS strains. *S. haemolyticus* has developed resistance to one or more antibiotics such as penicillin, cephalosporins, aminoglycosides, and glycopeptides. In recent years, advanced antibiotics such as vancomycin and teicoplanin have also been put into clinical use, due to the increasing resistance of *S. haemolyticus*. However, isolates with reduced susceptibility to vancomycin and teicoplanin have gradually emerged. In this case, vancomycin and teicoplanin were constantly administrated to patients. *S. haemolyticus* SH-1 was isolated from the patient and showed intermediate-resistance to vancomycin and teicoplanin. We speculate that the resistance of SH-1 could be related to the long-term administration of vancomycin and teicoplanin because the use of antimicrobial agents is often associated with the emergence of bacterial resistance.[20] However, the understanding of drug resistance of *S. haemolyticus* SH-1 is still unclear. Many resistance mechanisms have not been elucidated currently. In the present study, we focused on studying the mechanism underlying the reduced vancomycin susceptibility in *S. haemolyticus*.

For molecular epidemiology, ST97 *S. haemolyticus* is not the dominant clone and is relatively rare. Molecular type of SH-1 is the same as SYSUSHA_9 from Guangdong respiratory specimen in the database. However, VB19458, closer in evolution to SH-1, belongs to ST3 type. This result suggested that different clones are evolutionarily related to each other.[21] In Qin's study,[21] ST3 is the subdominant clone compared with the most emerging prevalent ST42 clone. Although ST97 is not the dominant clonal strain, MLST type is not a determining condition for the emergence of

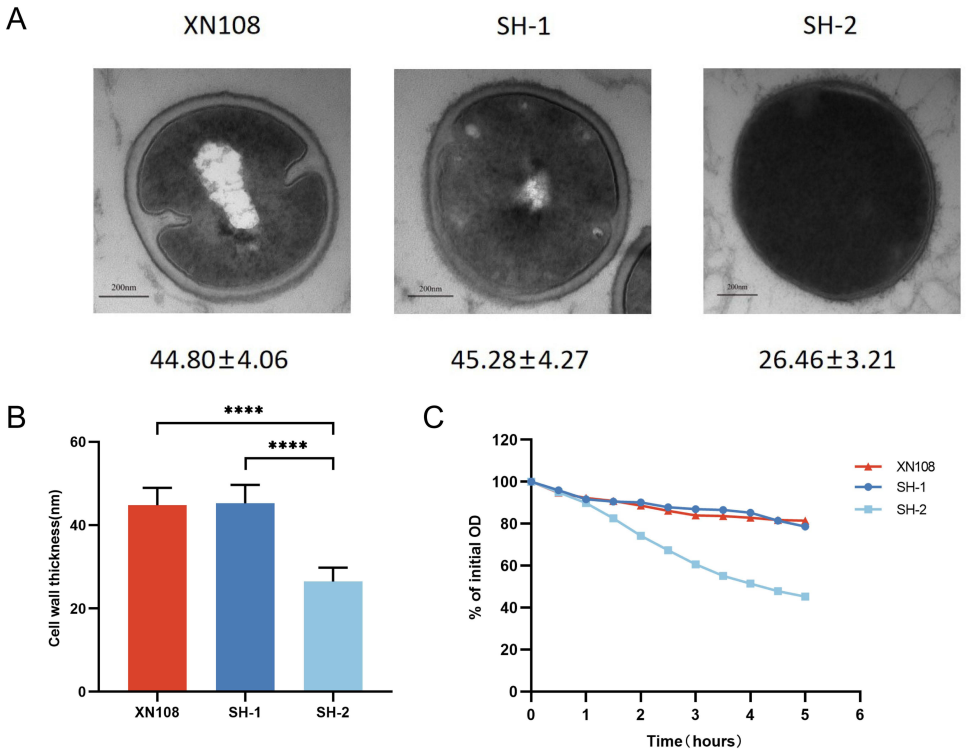

Figure 3 (**A**) Transmission electron microscopy images of representative cells of *S. haemolyticus* SH-1, SH-2 and S. aureus XN108. (**B**) Cell wall thickness histogram of different strains. (**C**) Autolytic activity detection of SH-1, SH-2, and XN108. Statistic significant difference calculated by the unpaired two-tailed *t*-test is indicated: ****P<0.0001.

VISH. ST97 strain SH-1 indicates that clinical endemic strains have capability to evolve resistance to vancomycin and vancomycin. Thus, the treatment with vancomycin and vancomycin may need to be minimized as much as possible in clinic.

Vancomycin is an electropositive glycopeptide antibiotic with the molecular weight of 1485 kDa. Vancomycin inhibits the reaction catalyzed by transglycosylase, transpeptidase, and carboxypeptidase in cell wall synthesis through a direct high-affinity binding to the D-alanyl-d-alanine at the terminal of the precursor of Staphylococcal peptidoglycan. It results in impaired cell wall synthesis, defection, and cell death.[22,23] VISA and VRSA have emerged clinically in *S. aureus*, which have different vancomycin resistance mechanisms. The emergence of VRSA is associated with the horizontal transfer of vancomycin resistance genes such as vanA,[24] but the spread of the resistance is more limited by the high adaptation cost of transposons.[25] In contrast, the mechanism of VISA formation is more complex and is associated with various factors such as cell wall thickening and mutations in regulatory genes.

In the study of VISA, the point mutations of two-component regulatory system of *S. aureus*, such as WalKR, GraSR,[26] VraSR,[27] AgrCA, SaeSR and ArlSR, may be associated with the acquisition of vancomycin intermediate-resistance type of clinical strains. Morever, mutations that present in RNA polymerase β subunit gene rpoB[28] and protein kinase gene stk1 are also contributed to the formation of clinical VISA isolates.[29] At present, some studies on vancomycin intermediate-resistant *S. aureus* have been reported while the investigation on the mechanism of *S. haemolyticus* is rare. According to CLSI,[13] the MIC of CoNS, such as *S. haemolyticus*, exhibiting a MIC≦4 μg/mL, indicates susceptibility to vancomycin, while intermediate-resistance to vancomycin is 8–16 μg/mL and complete resistance is MIC≧32 μg/mL. In our study, the MIC determined based on the E-Test method for SH-1 against vancomycin is 8 μg/mL, showing that SH-1 is intermediate-resistant to vancomycin. Whole genome sequencing reveals that SH-1 does not carry vancomycin complete resistance-related genes *vanA, vanB*, and *vanC*. We thus propose that the increase in vancomycin resistance in SH-1 may not be related to the mechanism of complete resistance.

Whole genome sequencing and SNP analysis showed that there were novel mutations of WalKR in *S. haemolyticus* SH-1. The two-component regulatory systems are important signal transduction regulatory pathways for bacterial adaptation to changes in multiple external stimuli.[30] Among these, WalKR is the only necessary TCS for the growth of *Staphylococci*. WalKR is also an important regulatory system involved in cell wall metabolism and cell division.[31] WalK(S221P), WalK(G223D), WalK(I237T), and WalR(K208R) mutations have been reported and proved that the histidine protein kinase WalK receives signals and is activated by autophosphorylation.[30,32,33] Then, the activated response regulator WalR regulates transcription of downstream target genes. In this process, mutations may affect the synthesis and autolytic activity of cell walls. Apart from the influence to phenotype, the mutations of WalKR may diminish the expression of a set of virulence factor genes,[30] and also decrease the hemolytic activity. Gardete et al[34] and Peng et al[35] reported that virulence-related genes such as *agrA, sarH1, spa*, and *lukD* were significantly down-regulated in VISA strains SG-R and XN108, respectively. By far, there are only a few studies on the molecular mechanism of reduced vancomycin susceptibility caused by WalKR mutations in *S. haemolyticus*. The WalK(N70K) and WalK(R280Q) mutations, the novel mutations of WalKR, identified in this study from clinical isolate SH-1 have not been reported in any *Staphylococcal* strains and may serve as a basis for molecular studies of vancomycin resistance.

Several studies have shown that vancomycin intermediate-resistant *Staphylococcus* exhibits some common characteristics including thickened cell walls,[36] decreased autolysis activity[37] and attenuated virulence.[38–40] In the previous studies, it was found that these phenotypes were relevant to the decreased susceptibility of vancomycin.[14,41–43] The gene expression of this kind of *Staphylococcus* has changed including the significantly down-regulated expression of cell wall metabolism genes, autolysin genes, virulence factor genes, and increased expression of cell wall biosynthesis genes.[44] The morphology of cell wall and autolytic activity was determined by transmission electron microscope (TEM) and Triton X-100-stimulated autolysis assays. The results showed that *S. haemolyticus* SH-1 exhibited typical thicken cell walls and decreased autolysis.

Conclusion

In summary, our study completed the whole genome sequencing, molecular typing, SNP analysis, and drug sensitivity analysis of a vancomycin intermediate-resistant *S. haemolyticus* SH-1, and determined the cell wall and autolysis activity aiming at the vancomycin intermediate-resistant phenotype. We supposed that the new WalKR point mutation could be contributed to the formation of vancomycin intermediate-resistance. The finding of the study may provide a basis for further understanding of the resistance mechanism of *S. haemolyticus* to vancomycin.

Ethics Statement

This study was approved by the ethics committee of the Third Affiliated Hospital of Guangzhou Medical University (approval number: 2021-035). All the personal information was removed and was not present in the data of this study. Written informed consent was obtained from patient.

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Disclosure

The authors declare no conflict of interest.

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